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RESEARCH PAPER

The *Arabidopsis* small GTPase AtRAC7/ROP9 is a modulator of auxin and abscisic acid signalling

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Abstract

Rac-like GTPases or Rho-related GTPases from plants (RAC/ROPs) are important components of hormone signalling pathways in plants. Based on phylogeny, several groups can be distinguished, and the underlying premise is that members of different groups perform distinct functions in the plant. AtRAC7/ROP9 is phylogenetically unique among 11 *Arabidopsis* RAC/ROPs, and here it was shown that it functions as a modulator of auxin and abscisic acid (ABA) signalling, a dual role not previously assigned to these small GTPases. Plants with reduced levels of AtRAC7/ROP9 had increased sensitivity to auxin and were less sensitive to ABA. On the other hand, overexpressing AtRAC7/ROP9 activated ABA-induced gene expression but repressed auxin-induced gene expression. In addition, both hormones regulated the activity of the AtRAC7/ROP9 promoter, suggesting a feedback mechanism to modulate the signalling output from the AtRAC7/ROP9-controlled molecular switch. High levels of AtRAC7/ROP9 were detected specifically in embryos and lateral roots, underscoring the important role of this protein during embryo development and lateral root formation. These results place AtRAC7/ROP9 as an important signal transducer in recently described pathways that integrate auxin and ABA signalling in the plant.

Key words: ABA, auxin, embryo development, hormone crosstalk, lateral roots, RAC/ROPs.

Introduction

The use of small GTP-binding proteins as important signal transducers is widespread in eukaryotes. They act as molecular switches, shuttling from a cytosolic inactive GDP-bound form to an active membrane-localized GTP-bound form. In this GTP-bound active form, the small GTPase interacts with other signalling components at the membrane, transducing the signal to effector molecules (VanAelst and DsouzaSchorey, 1997).

Plants have evolved a specialized class of small GTP-binding proteins called Rac-like GTPases or ROPs

(Rho-related GTPases from plants) (RAC/ROPs) that have emerged as crucial components of many signalling pathways (Berken, 2006; Nibau *et al.*, 2006; Yang and Fu, 2007; Yalovsky *et al.*, 2008). The activation status of RAC/ROPs is regulated by the activity of several upstream regulators. The plant-specific family of PRONE domain-containing guanine nucleotide exchange factors (ROPGEFs) and the DOCK domain-containing SPIKE1 promote RAC/ROP activation by stimulating GDP/GTP exchange (Berken *et al.*,

Abbreviations: ABA, abscisic acid; BAP, 6-benzylaminopurine; GA₃, gibberellic acid; GFP, green fluorescent protein; GUS, β-glucuronidase; LUC, luciferase; RAC/ROPs, Rac-like GTPases or Rho-related GTPases from plants; RLK, receptor-like kinase; ROPGEF, PRONE domain-containing guanine nucleotide exchange factors; NAA, 1-naphthylacetic acid; RNAi, RNA interference; SE, standard error; TIBA, 2,3,5-triiodo-benzoic acid.

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2005; Basu *et al.*, 2008). GTPase activating proteins accelerate GTP hydrolysis, converting them back to the inactive GDP-bound form and terminating signalling. Guanine nucleotide dissociation inhibitors negatively regulate protein activity by sequestering them in the cytosol, although they are also essential in their rapid recycling back to the site of activation on discrete plasma membrane locations (Yalovsky *et al.*, 2008). Several recent studies suggest that ROPGEFs might have evolved to transmit plant-specific signals from membrane-associated receptor-like kinases (RLKs). Two related leucine-rich repeat RLKs, the tomato LePRK2 and *Arabidopsis* AtPRK2a, were shown to interact with ROPGEFs during polarized pollen-tube growth (Wengier *et al.*, 2003; Kaothien *et al.*, 2005; Zhang and McCormick, 2007). More recently, a member of the *Catharanthus roseus* receptor-like kinase (CrRLK)-related family, FERONIA, was identified as an upstream activator of ROPGEFs during auxin-induced reactive oxygen species-mediated root hair development and regulates polar growth of pollen tubes and fungal hyphae (Duan *et al.*, 2010; Kessler *et al.*, 2010; Nibau and Cheung, 2011), and in maize epidermis, the receptor kinase PAN1 acts together with RAC/ROPs during stomatal cell division (Humphries *et al.*, 2011). Active RAC/ROPs can then promote signal diversification by binding a range of effectors leading to a plethora of responses, among them the regulation of cell shape and polar growth, hormone signal transduction, secondary cell-wall formation, defence responses against pathogens, responses to abiotic stresses, and light-mediated stomatal cell closure (Berken, 2006; Nibau *et al.*, 2006; Yang and Fu, 2007; Wu *et al.*, 2011).

Several RAC/ROPs have been implicated in hormone signalling. AtRac1 (AtRAC3/ROP6) acts as a negative regulator of abscisic acid (ABA) signalling in guard cells (Lemichiez *et al.*, 2001). RAC/ROPs such as the *Arabidopsis* AtRAC1/ROP3 regulate auxin-responsive gene expression by targeting the transcriptional regulators Aux/IAA proteins to proteolytic degradation by the proteasome, resulting in the derepression of auxin-responsive genes (Tao *et al.*, 2002, 2005). Besides auxin, AtRAC4/ROP2 and others have been shown to participate in ABA and brassinosteroid-regulated processes (Li *et al.*, 2001). Recently, a signalling pathway involving the GEF SPIKE1, ROP6, and the effector RIC1 was shown to regulate the localization and recycling of the auxin transporter PIN2 in roots (Lin *et al.*, 2012). In addition, ROP6 was shown to act downstream of the auxin-binding protein ABP1 to regulate the endocytosis of auxin transporters PIN1 and PIN2, regulating auxin transport during root and shoot development (Chen *et al.*, 2012). The characterization of a null mutant of AtRAC8/ROP10 and mutants that overexpressed active and inactive versions of this small GTPase revealed an important role for AtRAC8/ROP10 as a negative regulator of ABA signalling in *Arabidopsis* (Zheng *et al.*, 2002). More recently, AtRAC10/ROP11, most closely related to AtRAC8/ROP10 and speculated to have similar although not entirely overlapping functions, was also found to be a negative regulator of multiple ABA responses in *Arabidopsis* (Li *et al.*, 2012a; Yu *et al.*, 2012). In addition, auxin has been shown to activate RAC/ROPs in seedlings and protoplasts (Tao *et al.*,

2002; Xu *et al.*, 2010), while ABA inactivates AtRac1 in cell cultures (Lemichiez *et al.*, 2001).

Based on sequence analysis, RAC/ROPs can be further divided into types I and II (Winge *et al.*, 2000). The *Arabidopsis* type II AtRAC7/ROP9, AtRAC8/ROP10 and AtRAC10/ROP11 differ from type I by the presence of an extra exon at the 3' end of the gene. This results in loss of the C-terminal prenylation motif present in type I RAC/ROPs and, instead, the membrane association of type II RAC/ROPs requires *S*-acylation. The mechanism of *S*-acylation of type II RAC/ROPs is specific to plants and is responsible for RAC/ROP subcellular distribution, membrane interaction dynamics, and function (Sorek *et al.*, 2010, 2011). Interestingly, the four RAC/ROPs found in the moss *Physcomitrella patens*, are all type I, suggesting that type II RAC/ROPs have evolved to perform functions specific to land plants (Eklund *et al.*, 2010).

All the type II RAC/ROPs were initially placed in the same subgroup (Winge *et al.*, 2000; Zheng and Yang, 2000). The use of greater numbers of sequences and new phylogenetic analysis tools allowed Christensen *et al.* (2003) to further divide the type II RAC/ROPs into two groups, with AtRAC8/ROP10 and AtRAC10/ROP11 being placed in group 1 and AtRAC7/ROP9 being the only *Arabidopsis* RAC/ROP in group 2. This refined classification also suggests that members of the same group share similar functions that have been maintained throughout evolution (Christensen *et al.*, 2003). The same group structure was also preserved when *Physcomitrella* sequences were used (Eklund *et al.*, 2010).

Here, we report on the functional characterization of AtRAC7/ROP9 and suggest that it performs unique and specific functions within type II RAC/ROPs. AtRAC7/ROP9 was shown to have a distinctive expression pattern throughout the plant and this expression was regulated by developmental cues and hormones. Our results also showed that AtRAC7/ROP9 positively regulated ABA signalling while acting as a negative regulator of auxin signalling, and that it plays an important role in embryo and lateral root development. Our results suggest a model whereby AtRAC7/ROP9 acts as an integration point for auxin and ABA signalling, working to maintain the balance and sensitivity of these two signalling pathways in the plant.

Materials and methods

Chimaeric gene constructs and generation of transgenic plants

For the AtRAC7p-GUS and AtRAC10p-GUS constructs, a genomic fragment containing the 2 kb region upstream of the ATG of the genes was amplified by PCR and cloned upstream of the β -glucuronidase (GUS) gene in a pBluescript KS⁺ plasmid for protoplast expression or Ti-derived plasmid for plant transformation. The full-length AtRAC7/ROP9 cDNA was amplified from *Arabidopsis* seedling cDNA and cloned either downstream of its own promoter or of the cauliflower mosaic virus 35S promoter. For the AtRAC7/ROP9 RNA interference (RNAi) construct, a *Bgl*III/*Sal*I AtRAC7 cDNA fragment (nt 361–660) was cloned as an inverted repeat separated by a green fluorescence protein (GFP) sequence linker and cloned downstream of the 35S promoter. The DR5-GUS, 35S-LUC, UBI10-GUS, and rd29A-LUC constructs have been described previously (Ishitani *et al.*, 1997; Tao *et al.*, 2002). The Ti-derived

plasmid constructs were introduced into *Agrobacterium tumefaciens* strain GV2260 by conjugation and used to transform *Arabidopsis thaliana* Col. by the floral dip method (Clough and Bent, 1998). Kanamycin-resistant seedlings were selected and transferred to soil. Plants with a single insertion were identified by segregation analysis of the kanamycin marker, and homozygous lines were obtained and used for further analysis. *abil-1* seeds were obtained from the Arabidopsis Biological Resource Center, germplasm CS22.

RT-PCR analysis

Total RNA was isolated from 7-day-old *Arabidopsis* seedlings using an RNeasy Mini kit (Qiagen, Valencia, CA). Total RNA (2 µg) was used for reverse transcription reaction using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Two microliters of the reaction mixture was used as a template for the PCR. The RT-PCRs were repeated multiple times with similar results, and a representative result is shown.

Phenotypic analysis

Embryos were dissected from siliques and mounted in clearing solution, as described previously (Chen *et al.*, 2011). The cleared embryos were observed under differential interference contrast optics under an OLYMPUS BX51 microscope.

Seeds were surface sterilized and sown in plates containing basal medium [Gamborg B5 salts and vitamins, 2 mM MES (pH 5.6–5.8), 0.5 mM *myo*-inositol, 1% sucrose, 0.7% agar] or in soil (BX ProMix; Griffin Greenhouse Supplies, Tewksbury, MA) and kept at 4 °C for 3 d before transfer to growth chambers set at 21 °C under a 16 h light/8 h dark regime. Root length and lateral root numbers were determined in vertically grown seedlings. Representative results of multiple sets of experiments are shown. Seed dormancy was determined by plating freshly harvested seeds in medium containing ½ MS salts and vitamins, 1 mM MES (pH 5.6–5.8) and 0.7 % agar and incubating them at 21 °C without previous cold treatment. The percentage of germinated seeds (radicle emergence) was scored every day for 6 d. All experiments were repeated multiple times with comparable results, and representative results are shown. Student's *t*-test was used in all statistical analyses. Significance was defined as *P* < 0.05.

Hormone response assays

The effect of ABA on seed germination was determined by plating seeds in medium containing ½ MS salts and vitamins, and 1 mM MES (pH 5.6–5.8), supplemented with 0, 0.5, 1, or 1.5 µM ABA. After 3 d at 4 °C, plates were transferred to 21 °C and germination (radicle emergence) was scored 3 d after transfer. *abil-1* seeds (germplasm CS22) were used as a control. For determining the responsiveness to auxin induction of lateral root formation, the number of lateral roots was determined in medium containing 0, 25, or 50 nM 1-naphthylacetic acid (NAA). For determining the responsiveness to auxin, ABA, 6-benzylaminopurine (BAP), and epi-brassinolide inhibition of root growth, seeds were plated in basal medium without hormones, cold treated for 3 d, and incubated vertically at 21 °C for 5 d. After this period, the seedlings were transferred to basal medium supplemented with hormones, as indicated in the figures, and the position of the primary root tip was marked. Growth in the hormone-supplemented medium was measured after 4 d. The percentage of root growth relative to seedlings grown in medium without hormone was calculated. The effect of gibberellins and auxin on hypocotyl elongation was determined as described previously (Smalle *et al.*, 1997).

All measurements were performed using Scion Image software (Scion Corporation, MD) from digital images. All experiments were repeated multiple times with comparable results. Unless otherwise described in the figures, results shown are means ± standard error (SE)

of three replicates with ten seedlings each. Student's *t*-test was used in all statistical analyses. Significance was determined as *P* < 0.05.

Histochemical GUS assays and GFP imaging

Seedlings from several lines homozygous for the AtRAC7p–GUS construct were collected at different stages of development and stained for GUS activity. Most lines showed similar patterns of expression, and a representative line was selected for detailed analysis. The GUS reaction was allowed to develop at 37 °C for 12–16 h unless otherwise noted. After staining, seedlings were cleared in 70% ethanol and imaged. Developing seeds from fertilized pistils were used for GUS activity assays in developing embryos following the method of Weijers *et al.* (2001). For assays of 2,3,5-triiodo-benzoic acid (TIBA) inhibition of lateral root formation, AtRAC7p–GUS seedlings were grown vertically in basal medium or in medium containing 1 µM TIBA for 3 d. After this period, the seedlings were transferred to basal medium and incubated vertically. At 12, 24, 36, and 48 h after transfer, the seedlings were collected and used for GUS histochemical staining. For determination of hormone effects on AtRAC7/ROP9 and AtRAC10/ROP11 promoter activity, seedlings were grown in liquid basal medium for 5 d. After this period, the medium was replaced by medium containing the following components: no hormone, 1 µM NAA, 10 µM ABA, 1 µM gibberellic acid (GA₃), or 1 µM BAP, and incubated for 24 h. The same assays were performed with agar-grown seedlings with comparable results. GFP–AtRAC7 protein localization was observed in intact roots or transfected protoplasts by epifluorescence microscopy (Nikon Eclipse E800).

Protoplast transfection, and GUS and luciferase (LUC) assays

Protoplasts were isolated from 3-week-old tissue-culture-grown *Arabidopsis* seedlings and transfected as described previously (Tao *et al.*, 2002). Typically 0.2 ml of cells (2 × 10⁶ cells ml⁻¹) were transfected with 10 µg of the reporter gene construct and 5 µg of the 35S–LUC or Ubi10–GUS construct as an internal control for transfection efficiency for each transformation. Medium supplemented with hormones as indicated in the figures was added after transfection. Protoplasts were incubated for 12–16 h in the dark, harvested, and used for GUS and LUC assays as described previously (Tao *et al.*, 2002). Each assay was done in triplicate and each experiment was repeated at least three times.

Accession numbers

The Arabidopsis Information Resource accession numbers for the genes determined in this study are: At4g28950 (AtRAC7/ROP9), At3g48040 (AtRAC8/ROP10), and At5g62880 (AtRAC10/ROP11).

Results

AtRAC7/ROP9 has an expression pattern distinct from other type II RAC/ROPS

In order to characterize the spatial and temporal expression of AtRAC7/ROP9 in *Arabidopsis*, transgenic plants carrying a fusion of the AtRAC7/ROP9 promoter and the GUS gene (AtRAC7p–GUS) were generated. The AtRAC7p–GUS lines obtained were stained for GUS activity at different stages of development and a representative line was used for detailed characterization. Contrary to what was observed previously for AtRAC8/ROP10 (Zheng *et al.*, 2002) and AtRAC10/ROP11 (Li *et al.*, 2012a; Yu *et al.*, 2012), where both genes were expressed in the majority of the tissues tested, including stomata and pollen, the pattern for AtRAC7/ROP9 expression

was considerably more discrete (Fig. 1). AtRAC7/ROP9p activity was detected in the developing embryo (Fig. 1A–C) and in the young seedlings (Fig. 1D). Later in development, GUS expression became restricted to the shoot and root meristems (Fig. 1E) and the vascular system (Fig. 1F); no expression was detected in female or male reproductive tissues or in stomata (Fig. 1G, H). The expression pattern of AtRAC7/ROP9 in the root meristem and in the lateral root primordia was striking (Fig. 1E, I, J). Promoter activity was detected at the lateral root primordia (Fig. 1I), and as the root developed it became restricted to the meristem (Fig. 1J).

To determine how early in lateral root development AtRAC7/ROP9 is expressed, a lateral root-inducible system based on treatments with an auxin transport inhibitor was used (Himanen *et al.*, 2002). Auxin transport inhibitors have been shown to prevent the formative divisions in the pericycle that lead to lateral root initiation (Casimiro *et al.*, 2001). When AtRAC7p–GUS seedlings were grown in basal

medium for 3 d, staining was observed in the dividing cells giving rise to the lateral root primordium and 48 h later in the apical meristem of well-developed lateral roots (Fig. 2A). On the other hand, AtRAC7p–GUS seedlings grown for 3 d in medium supplemented with 1 μ M TIBA did not show any GUS-positive lateral root primordia, consistent with lateral root initiation being inhibited. These seedlings were transferred to basal medium and tested for GUS at 12 h intervals. GUS activity was detectable only in the apical root meristem up to 24 h after transfer and no lateral root initiation sites were visible in these TIBA-treated plants (data not shown). Between 24 and 48 h after transfer, the first lateral root initiation sites became visible and AtRAC7/ROP9 promoter activity was detected when the first divisions leading to lateral root primordia formation were occurring (Fig. 2A). In addition, GUS expression was detected along the whole pericycle layer when auxin was applied to AtRAC7p–GUS seedlings (Supplementary Fig. 1A at JXB online). These observations

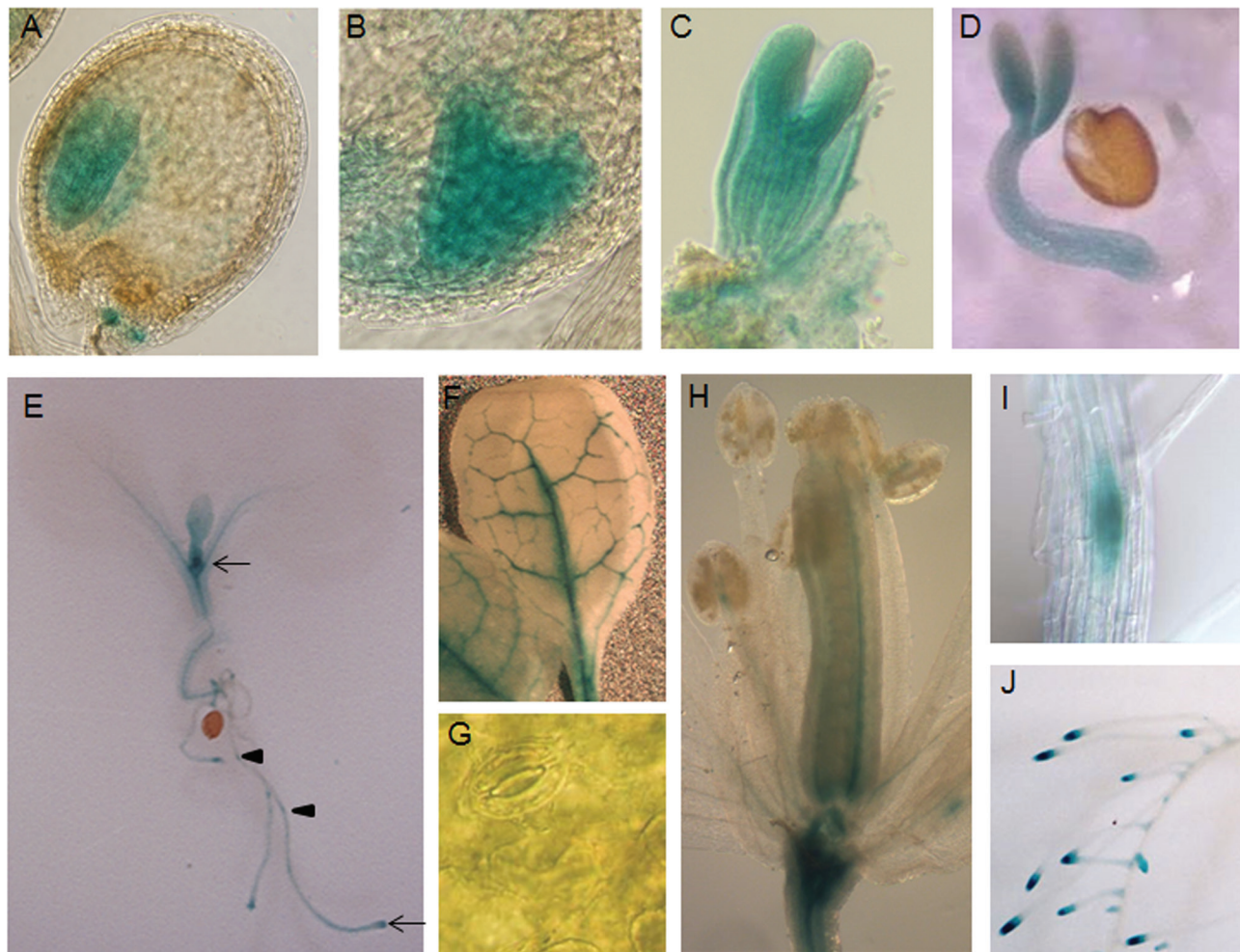


Fig. 1. Developmental expression pattern of the AtRAC7/ROP9 promoter in *Arabidopsis* as observed by histochemical GUS staining. AtRAC7/ROP9p–GUS expression was detected in the developing embryo (A–C) and in the germinating seedling (D). In young seedlings, activity was detected in the meristematic region (arrow), lateral root primordia (arrow heads) (E) and in the vascular system (F). No AtRAC7/ROP9 expression was detected in stomata (G). In female and male reproductive organs, expression was detected only in the vascular system (H) and, notably, no expression was detected in pollen. High levels of AtRAC7/ROP9 expression were observed in the developing lateral roots (I) and in the meristem of elongating lateral roots (J).

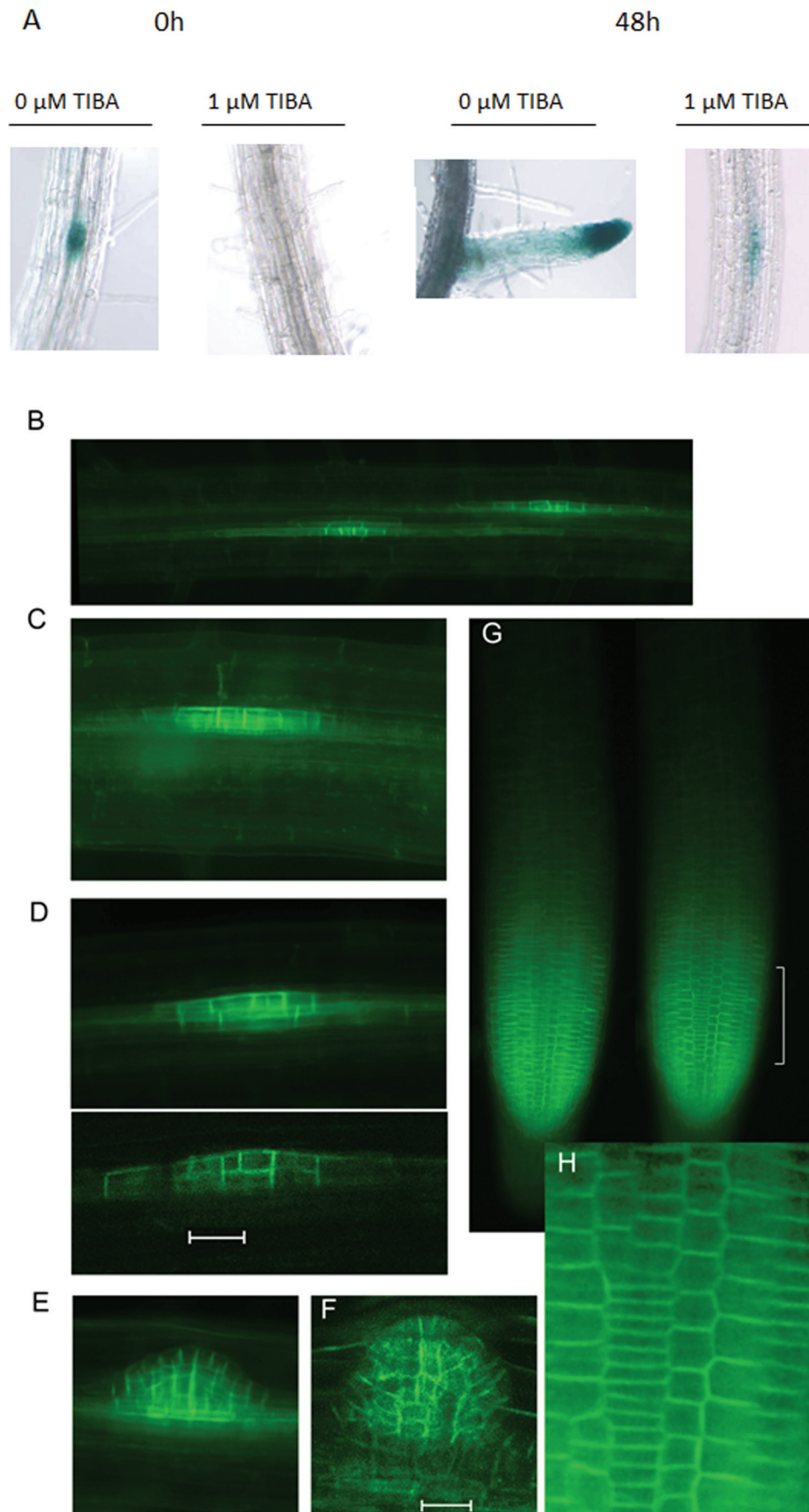


Fig. 2. AtRAC7/ROP9 is expressed in the early stages of lateral root formation. (A) Activity of the AtRAC7/ROP9 promoter was studied in a lateral root-inducible system. AtRAC7p-GUS seedlings were grown for 3 d in medium containing 0 or 1 μ M TIBA (pre-treatment). After this period, some seedlings were stained for GUS activity (0h) and the remaining seedlings were transferred to medium without

are consistent with AtRAC7/ROP9 being transcriptionally upregulated during auxin-induced lateral root initiation in *Arabidopsis* using microarray analysis (Vanneste *et al.*, 2005) and further illustrate the fact that the AtRAC7/ROP9 promoter is only active in specific cell types in developing seedlings, even under auxin-activated conditions.

In transformed plants grown under normal conditions, low levels of GFP–AtRAC7/ROP9 expressed from the AtRAC7/ROP9 promoter could be detected in the pericycle cells as early as the first divisions that give rise to the primordium, and continued to be restricted to the primordium as it developed (Fig. 2B–F). In the primary root, GFP–AtRAC7/ROP9 was only observed at the root tip (Fig. 2G, H). Furthermore, and unlike type I RAC/ROPs whose inactive and activated forms partition into the cytoplasm and the plasma membrane, respectively, the GFP–AtRAC7/ROP9 protein was found to be constitutively associated with the plasma membrane in root cells and in transfected protoplasts (Fig. 2D, lower panel, and H, Supplementary Fig. S1B).

Downregulation of the AtRAC7/ROP9 gene causes embryo patterning defects

The distinctive pattern of expression of the AtRAC7/ROP9 gene prompted a more detailed investigation of the functions of the AtRAC7/ROP9 protein in plants. None of the available T-DNA insertion mutants resulted in plants with reduced levels of AtRAC7/ROP9 mRNA (data not shown). To circumvent this problem, we used RNAi technology to generate plants with downregulated levels of AtRAC7/ROP9. A fragment of the AtRAC7/ROP9 cDNA at the 3' end (nt 361–660) was chosen to make the construct because it is the most variable region between the *Arabidopsis* RAC/ROP genes. Compared with other transformations, we experienced a considerably higher level of lethality among T1-transformed plants selected from several independent transformation experiments. Many showed seedling defects resulting in their failing to grow to maturity. Of the 68 independent lines recovered, we were unable to find a line with no detectable levels of AtRAC7/ROP9 mRNA. Of the homozygous lines that showed substantially reduced levels of AtRAC7/ROP9 mRNA, we chose the Ri-6, Ri-10, and Ri-11 lines for further analysis (Fig. 3A). The mRNA levels of other RAC/ROPs, including those of the most related AtRAC8/ROP10 and AtRAC10/ROP11 and of the type I AtRAC3/ROP6, were not affected in these lines (Fig. 3A). Among the progeny, we observed some developmental defects (Fig. 3B). An increased rate of seedlings showed arrested primary root growth (Table 1 and

Fig. 3B), and there was a small but significant increase in the number of seedlings where the primary root did not develop and in those with defective cotyledons (Table 1). We always observed a range of phenotypes (from severe to mild) in the progeny from homozygous RNAi lines, reflecting the fact that these lines still maintained some AtRAC7/ROP9 activity or that other compensatory pathways were activated (Fig. 3B).

To look at the embryo phenotype in more detail, progeny seeds from homozygous plants were analysed to determine the prevalence of defects. As can be seen in Fig. 3C, some of the embryos of both RNAi lines showed abnormal cell divisions in both the embryo proper and suspensor at the 16- and 32-cell stage (Fig. 3C, first two columns). In globular- and triangular-stage embryos (Fig. 3C, last two columns), we observed atypical cell divisions in the hypophyseal cell region (arrows and brackets). The rate of abnormal embryos in the line Ri-6 was 5% ($n=126$) and in Ri-11 was 5.8% ($n=165$), whereas no abnormal embryos were detected in the wild type ($n=178$). The embryo defect frequency was comparable to that detected at early embryogenesis for plants with reduced levels of ROPGEF7 (Chen *et al.*, 2011) and was most likely the combined result of residual AtRAC7/ROP9 protein in these lines and the presence of other RAC/ROPs that compensate for the reduced AtRAC7/ROP9 during embryo development. Although occurring at a lower frequency, the embryo defects observed in the AtRAC7/ROP9 RNAi lines also resembled the phenotypes of other auxin signalling mutants, such as the auxin receptor mutant *tir1 afb1 afb2 afb3* (Dharmasiri *et al.*, 2005) and root master regulator mutants *plt1 plt2 plt3* and *plt1 plt3 bbm-2* (Galinha *et al.*, 2007). In these *plt* mutants, the effect of gene redundancy was also evident: single *plt1* or *plt2* mutants did not show an embryo phenotype, while in the double mutant *plt1 plt2*, some basal embryo defects were observed (Aida *et al.*, 2004) and only disruption of all three *PLT* genes led to a severe embryonic phenotype with high ratio of defective embryos (Galinha *et al.*, 2007). The early embryo defects observed in the embryo proper and abnormal cell organization in hypophyseal region in the RNAi lines probably caused some of the root phenotypes observed in seedlings (Fig. 3B). In addition, the root phenotypes observed later in development where primary root growth was arrested may have resulted from a post-embryonic effect of AtRAC7/ROP9. In soil, the plants that survived to maturity were indistinguishable from the wild type except that they were found to flower slightly but significantly earlier than wild-type plants (Supplementary Fig. S2C at JXB online). Primary root growth and lateral root numbers of the RNAi seedlings were not significantly different from the wild type (Supplementary Fig. S2A, B).

TIBA and stained for GUS activity 48 h after transfer. (B–H) GFP–AtRAC7/ROP9 is specifically detected at the plasma membrane of the pericycle cells that divide to form the lateral root primordia. (B–D) lateral root initials; (E, F) emerging lateral roots; (G) two focal planes of a primary root showing GFP–AtRAC7/ROP9 is only detected at the root tip; bracket region magnified in (H). (D) lower panel, a single confocal plane; (F) maximum projection from serial confocal sections; scale bar = 50 μ m.

Downregulation of AtRAC7/ROP9 results in decreased responsiveness to ABA

To determine whether the AtRAC7/ROP9 protein participates in hormone signalling pathways, the RNAi lines were further characterized for their sensitivity to different exogenously applied hormones. First, we tested their responses to ABA, which is known to inhibit seed germination and root

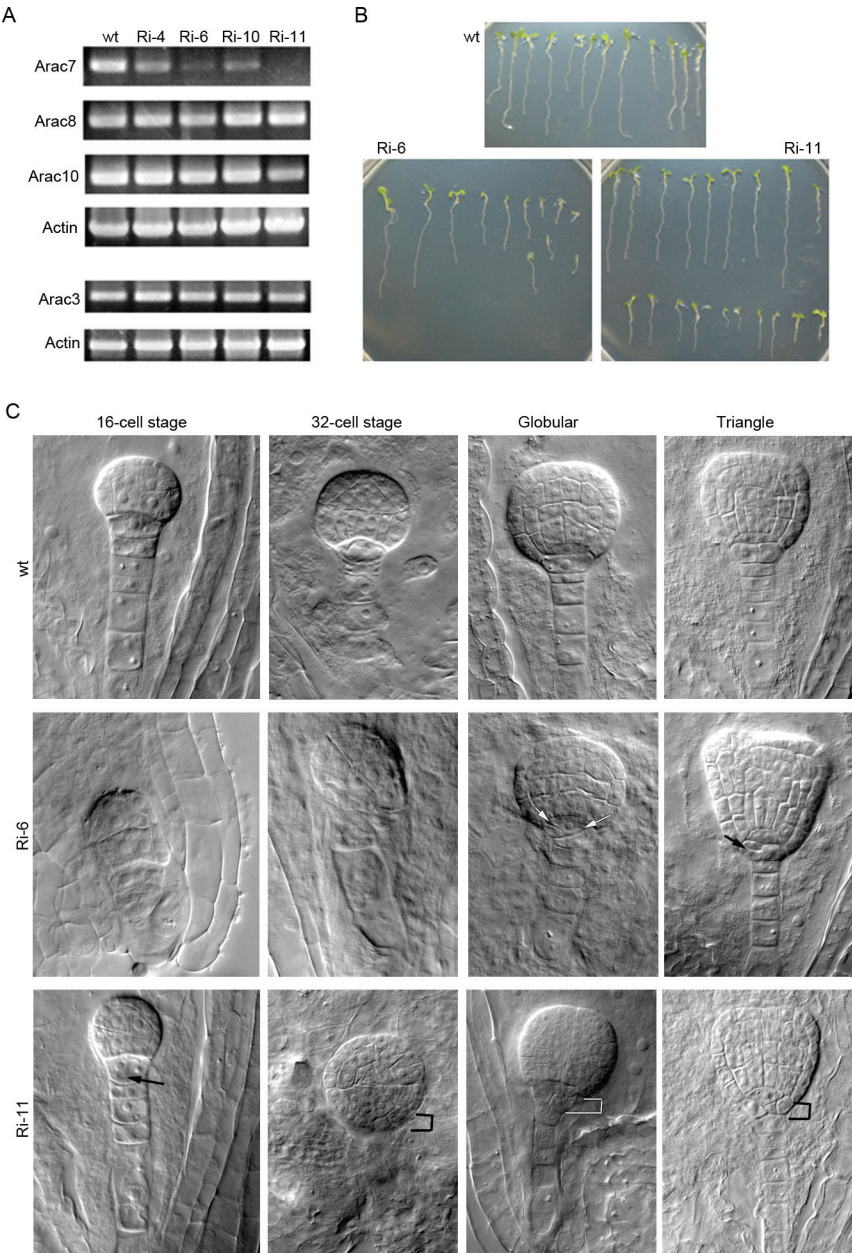


Fig. 3. Reduced levels of AtRAC7/ROP9 mRNA result in developmental defects. (A) RT-PCR analysis of AtRAC7/ROP9 expression levels in wild-type plants and selected AtRAC7/ROP9 RNAi (Ri) lines. Ri-6, Ri-10, and Ri-11 had substantially reduced levels of AtRAC7/ROP9 mRNA, while AtRAC8/ROP10, AtRAC10/ROP11, and AtRAC3/ROP6 mRNA levels were not affected in these lines. Actin was used as an internal control. (B) Growth defects in 8-day-old vertically grown seedlings observed among the progeny of the lines in (A). (C) Embryo defects observed in plants with reduced levels of AtRAC7/ROP9. From left to right, 16-cell stage, 32-cell stage, and globular and triangular stages. Aberrant divisions of cells in the hypophyseal region of embryos are indicated by arrows and brackets, indicating that single and multiple aberrant divisions occurred.

Table 1. Percentage of seedling defects among wild-type and AtRAC7/ROP9 RNAi progeny

	Short root	No root	Cotyledon defects
Wild type	5.71% ($\pm 1.50\%$)	0.25% ($\pm 0.43\%$)	0.15% ($\pm 0.26\%$)
Ri-6	34.84% ($\pm 3.17\%$)*	1.43% ($\pm 0.17\%$)*	1.40% ($\pm 0.32\%$)*
Ri-11	38.15% ($\pm 6.05\%$)*	4.11% ($\pm 0.49\%$)*	1.84% ($\pm 0.72\%$)*

*Values are means \pm standard deviation ($n > 100$) and significantly different for the wild type at $P < 0.05$.

elongation. Seeds from the RNAi lines were less responsive to ABA inhibition of germination (Fig. 4A). Seeds from *abi1-1*, a mutant whose germination is insensitive to exogenous ABA (Koornneef *et al.*, 1984), were used as a positive control for this experiment. ABA also increases seed dormancy, preventing germination under adverse conditions. Freshly harvested seeds were allowed to germinate without previous cold treatment. As can be seen in Fig. 4B (solid lines), AtRAC7/ROP9 RNAi seeds germinated faster than the wild type, implying that downregulation of AtRAC7/ROP9 gene expression decreased

seed dormancy. When cold treated to break dormancy, the two lines germinated at similar rates (Fig. 4B, dashed lines). The observed effects of decreased levels of AtRAC7/ROP9 on seed dormancy and germination correlated well with AtRAC7/ROP9p activity being detected in embryos (Fig. 1A–C). Seedlings from AtRAC7/ROP9 RNAi lines were found to be less sensitive to ABA inhibition of root elongation over a wide range of tested concentrations (Fig. 4C). Further supporting the role of the AtRAC7/ROP9 protein as a positive regulator of ABA signalling, overexpression of AtRAC7/ROP9 in transiently transfected protoplasts increased transcription from an ABA signalling-responsive promoter, rd29A (Fig. 4D).

Plants with downregulated levels of AtRAC7/ROP9 expression show increased responses to auxin

The response of the RNAi lines to auxin was also tested. The suggestion that the auxin signalling pathway might

be disturbed in these plants came from the observation of defective embryos. Auxin distribution and signalling are crucial for the regulation of cell division and patterning during embryo development. Moreover, auxin is an important regulator of lateral root formation and the ability of auxin, when applied exogenously, to induce lateral root formation is often used in assays to monitor auxin responses in the plant. When grown in medium containing 25 or 50 nM of the auxin NAA, the RNAi lines were found to produce significantly more lateral roots than the wild type (Fig. 5A) and this effect was observed over an extended period of time (Fig. 5B). Another well-known effect of auxin is the inhibition of root elongation. In these assays, Ri-6 and Ri-11 seedlings were found to be more sensitive to inhibition of root elongation at low auxin concentrations (Fig. 5C). At higher concentrations, the effect was less pronounced, and only Ri-6 showed a small but significant difference from wild type (Fig. 5C). Consistent with plants having reduced levels of AtRAC7/

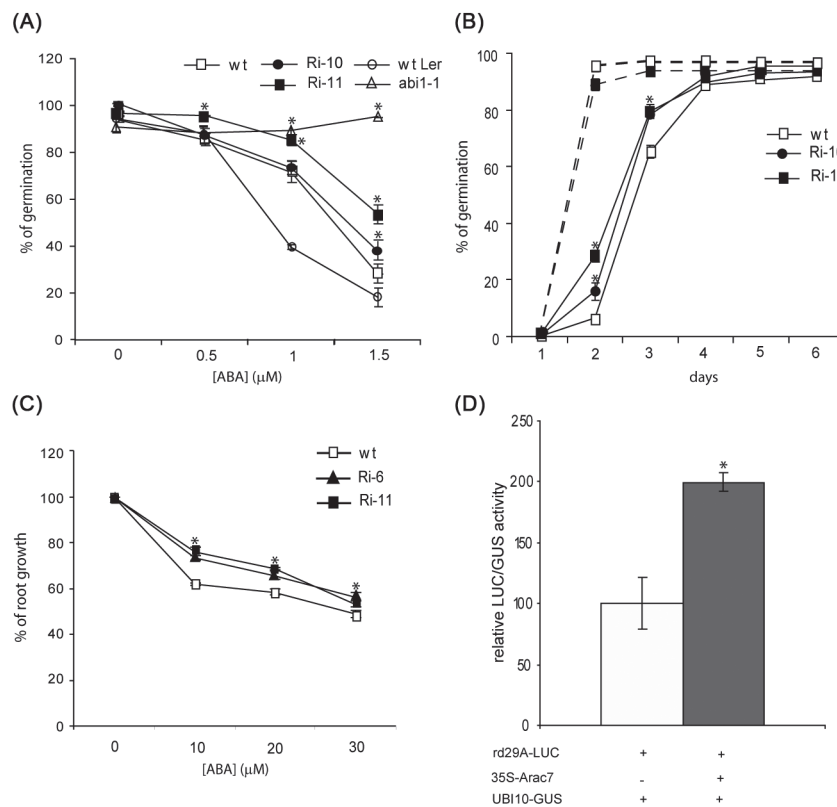


Fig. 4. AtRAC7/ROP9 acts as a positive regulator of ABA signalling in *Arabidopsis*. (A) AtRAC7 RNAi seeds from AtRAC7/ROP9 RNAi lines are less sensitive to ABA inhibition of seed germination. Seeds were plated in medium containing the indicated ABA concentrations and germination (percentage germinated/total seeds) was determined after 3 d. Data represent means \pm SE of three replicates with \sim 100 seeds each. The ABA-insensitive mutant *abi1-1* in the Landsber erecta (Ler) background was used as a positive control. (B) Freshly harvested seeds from AtRAC7/ROP9 RNAi lines show decreased dormancy when compared with wild type seeds. Solid lines represent seeds allowed to germinate without previous cold treatment, with germination scored every day from day 1 to day 6. Dashed lines represent the germination percentage of the same seeds cold treated for 3 d before transfer to the growth chamber. Data represent means \pm SE of three replicates with \sim 100 seeds each. (C) Seedlings with reduced levels of AtRAC7/ROP9 are less sensitive to ABA inhibition of root elongation. Root elongation in the ABA medium was calculated as a percentage of growth in medium without the hormone. Data represent means \pm SE of three replicates with ten seedlings each. (D) Overexpression of AtRAC7/ROP9 stimulates transcription from an ABA-inducible rd29A promoter in transiently transformed protoplasts. An asterisk indicates values significantly different from the wild type grown under the same conditions for $P < 0.05$.

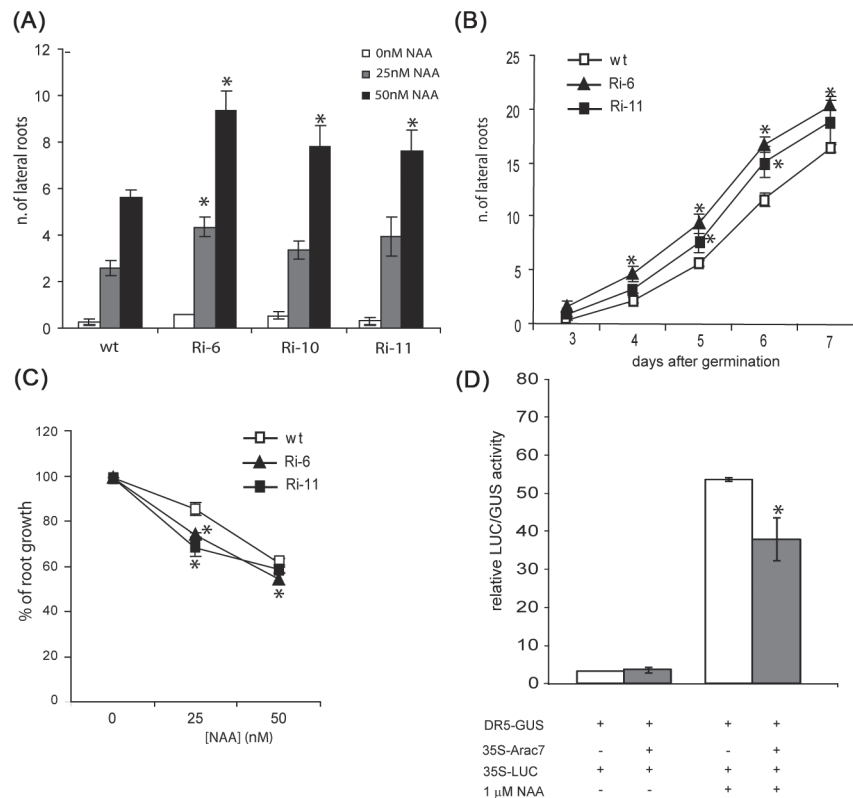


Fig. 5. AtRAC7/ROP9 is a negative regulator of auxin signalling in *Arabidopsis*. (A) Lateral root numbers of 5-day-old wild type, Ri-6, Ri-10, and Ri-11 seedlings grown in basal medium or basal medium supplemented with 25 or 50 nM NAA. Bars represent means \pm SE of three replicates with ten seedlings each. (B) Lateral root numbers of seedlings grown in 50 nM NAA determined from day 3 to day 7 after germination. Data represent means \pm SE of three replicates with ten seedlings each. (C) Responsiveness of wild type, Ri-6, and Ri-11 seedlings to root growth inhibition by auxin. Three-day-old seedlings were transferred to medium containing 25 or 50 nM NAA. The percentage of root growth in this medium was calculated relative to medium with no hormone. Bars represent means \pm SE of three replicates with ten seedlings each. (D) AtRAC7/ROP9 represses auxin-induced gene expression in transfected protoplasts. Protoplasts were transfected with the reporter gene DR5-GUS with or without the effector construct 35S-AtRAC7 and cultured in the presence or absence of exogenous auxin as indicated. An asterisk indicates values significantly different from the wild type grown under the same conditions at $P < 0.05$.

ROP9 showing increased auxin responses, overexpression of AtRAC7/ROP9 in protoplasts repressed auxin-induced activity of the auxin-responsive DR5 promoter (Fig. 5D). Taken together, these data suggested that AtRAC7/ROP9 plays a role as a negative modulator of auxin signalling pathways.

The effect of decreased levels of AtRAC7/ROP9 in other hormone pathways was also tested. Induction of hypocotyl elongation in response to the application of the gibberellin GA₃ in the AtRAC7/ROP9 RNAi seedlings was comparable to that of wild type (Supplementary Fig. S2D). Interestingly, the RNAi lines showed increased responsiveness to auxin induction of hypocotyl elongation when auxin was applied alone or together with GA₃ (Supplementary Fig. S2D), consistent with the previous observations that plants with downregulated levels of AtRAC7/ROP9 were more responsive to auxin. The RNAi lines showed the same level of responsiveness to the inhibition of root elongation by the cytokinin BAP and by epi-brassinolide (Supplementary Fig. S2E, F).

ABA and auxin regulate transcription from the AtRAC7/ROP9 promoter

To elucidate how AtRAC7/ROP9 expression levels are regulated in the plant, the AtRAC7p-GUS transgenic lines were exposed to different hormonal signals. As can be seen in Fig. 6A, the activity of the AtRAC7/ROP9 promoter was regulated by hormones. Exogenous application of auxin stimulated promoter activity, while ABA was a strong inhibitor. The application of other hormones did not have an effect on the AtRAC7/ROP9p activity (Fig. 6A). The results obtained with the histochemical staining were extended using a transient expression assay in protoplasts, which showed that auxin stimulated the activity of the AtRAC7/ROP9 promoter, while ABA was a strong repressor (Fig. 6B). The dual regulation of the AtRAC7/ROP9 promoter by auxin and ABA is unique among type II RAC/ROPs, as auxin had no effect on the AtRAC10/ROP11 promoter (Supplementary Fig. S3 at JXB online) or the AtRAC8/ROP10 promoter (Zheng *et al.*, 2002), while ABA was a strong repressor of both promoters.

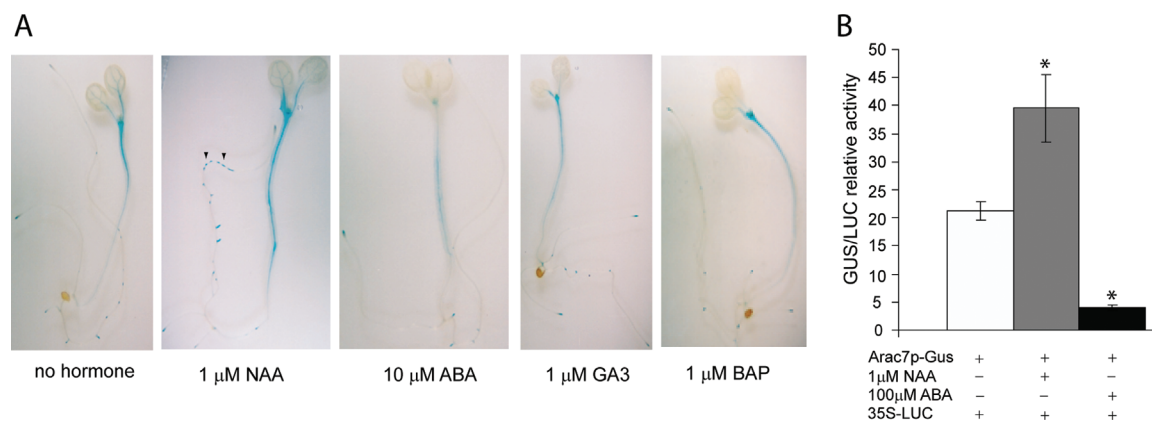


Fig. 6. Effect of different hormones on the activity of the AtRAC7/ROP9 promoter. (A) Five-day-old liquid-grown AtRAC7p-GUS seedlings were incubated in medium with no hormone, 1 μ M NAA, 10 μ M ABA, 1 μ M GA, or 1 μ M BAP for 24 h before GUS staining. Arrowheads indicate an area of auxin-induced GUS-positive lateral root primordia. (B) Effect of auxin and ABA on the activity of the AtRAC7/ROP9 promoter in transiently transfected protoplasts. *Arabidopsis* protoplasts were transfected with the AtRAC7p-GUS construct and incubated in the presence or absence of hormones, as indicated. Data represent means \pm standard deviation of three replicates. An asterisk indicates values significantly different at $P < 0.05$.

Discussion

The explosion of knowledge in recent years on how hormone signals are perceived and transduced in the plant has revealed a complex network of pathways that give rise to multiple responses that together shape development and adaptation to the environment. It is apparently a balancing act that the plant maintains sensitivity to multiple signals while integrating them all to produce an appropriate response (Moller and Chua, 1999). However, little is known about the identity of the integration points for the various signalling pathways. The opposing effects shown here for how AtRAC7/ROP9 regulates auxin and ABA signalling and how these hormones in turn regulate AtRAC7/ROP9 promoter activity suggest that this small GTPase plays a role in the coordination of the two hormone signalling pathways.

Considering that a large number of AtRAC7/ROP9 RNAi T1 plants, which were produced at a normal and relatively high transformation frequency in several independent transformation experiments, did not survive past the plantlet stage, and the fact that none of the recovered transformed plants was entirely devoid of AtRAC7/ROP9 mRNA, it seems reasonable to suggest that completely abolishing AtRAC7/ROP9 expression is detrimental for development. In addition, embryogenic defects were observed among the progeny plants with reduced levels of AtRAC7/ROP9, consistent with its expression being detected in developing embryos (Figs 1 and 3). Auxin is crucial for embryo patterning and development, and many mutants with altered auxin signalling show embryogenic defects (Jenik and Barton, 2005). It is thus likely that the effect of AtRAC7/ROP9 downregulation on embryo development is a manifestation of altered auxin signalling pathways. Further supporting this suggestion is the observation that, in seedlings, reducing AtRAC7/ROP9 levels resulted in increased sensitivity to auxin during root growth, lateral root formation, and hypocotyl elongation, while overexpression of AtRAC7/ROP9 repressed auxin-induced gene

expression (Fig. 5). Due to the important role of auxin transport and distribution during embryo development (Jenik and Barton, 2005) and the involvement of some AtRAC/ROPs in the localization of auxin transporters in *Arabidopsis* (Lin et al., 2012; Nagawa et al., 2012); the effect of AtRAC7/ROP9 during embryo development may also be due to altered auxin transport in the embryo or a more complex interaction between auxin transport and signalling.

The pattern of expression of AtRAC7/ROP9 in roots is unique among the *Arabidopsis* RAC/ROPs examined thus far (Figs 1 and 2). In the primary root, expression was detected in the apical meristem area and in lateral root primordia where expression was detected as early as the first pericycle divisions, a step known to be auxin dependent (Nibau et al., 2008). Moreover, AtRAC7/ROP9 is unique in its role as a negative regulator for auxin signalling, distinct from those RAC/ROPs that positively regulate auxin-mediated responses (Tao et al., 2002, 2005) and from the other two most closely related type II RAC/ROPs, which are specific negative regulators of ABA signalling and do not affect auxin signalling (Li et al., 2012a; Zheng et al., 2002).

In the initial phylogenetic classifications, AtRAC7/ROP9, AtRAC8/ROP10, and AtRAC10/ROP11 were placed in the same subgroup, leading to the suggestion that they would function redundantly (Zheng et al., 2002). Using more sequences and a more stringent analysis, AtRAC7/ROP9 has been placed in a different subgroup (Christensen et al., 2003). Our data clearly showed that AtRAC7/ROP9 is a positive regulator of ABA signalling during seed germination, dormancy maintenance, and root elongation (Fig. 4). Both AtRAC8/ROP10 and AtRAC10/ROP11 regulate ABA-mediated stomatal closing in *Arabidopsis* leaves (Li et al., 2012b; Zheng et al., 2002), while AtRAC7/ROP9 is not expressed in guard cells and its downregulation in the RNAi lines had no effect on ABA-induced water loss (Fig. 1G, and data not shown). These data provide functional support for AtRAC7/ROP9 being in

its own subgroup among *Arabidopsis* RAC/ROPs and further strengthen the suggestion that closely related RAC/ROPs generally display similar expression patterns and control the same biological processes, while RAC/ROPs members without homologues in the same group typically have unique expression patterns and possibly unique functions (Eklund *et al.*, 2010). Interestingly, the expression pattern of the maize Rop3, -6, and -7, included in the same group as AtRAC7/ROP9, is also different from the other maize type II RAC/ROPs (Christensen *et al.*, 2003). The different groups can also be distinguished based on the analysis of consensus amino acid sequences at the N terminus located in regions involved in effector binding, and AtRAC7/ROP9 has clusters of amino acid substitutions in this region, further supporting the suggestion that this small GTPase provides a specific biological need not met by other members of the *Arabidopsis* RAC/ROP family.

We propose that AtRAC7/ROP9 acts at the interface of auxin and ABA signalling, integrating the typically opposing responses to these two hormones. Exogenously applied auxin induces transcription from the AtRAC7/ROP9 promoter, while ABA is a strong repressor (Fig. 6), and the presence of *cis* elements used in auxin- and ABA-regulated transcription in its promoter (Supplementary Fig. S4 at JXB online) suggest that the signalling capacity of AtRAC7/ROP9 could be modulated by these hormones endogenously. Although the majority of publicly available microarray data do not show an effect of these hormones on the levels of AtRAC7/ROP9, this could be due to the fact that these studies used whole seedlings, and AtRAC7/ROP9 is only expressed in specific tissues, as revealed by AtRAC7/ROP9p–GUS analysis (Fig. 1).

Our observations suggest the existence of a feedback loop that regulates AtRAC7/ROP9 expression and at the same time is responsible for the modulation of auxin and ABA signalling, thus maintaining the cell's sensitivity to the hormone signals (Supplementary Fig. S5). We propose that auxin sensing triggers a number of auxin-related responses, and at the same time transcription of AtRAC7/ROP9 is induced, which in turn will negatively regulate the auxin pathway and thus reset it. The increased levels of AtRAC7/ROP9 will have a positive effect on the ABA signalling pathways. ABA signalling represses AtRAC7/ROP9 expression, thereby causing down-regulation of its own pathway. Together they provide the means for the cell to maintain responsiveness to ABA and at the same time derepress auxin signalling. At any given time, cells are exposed to multiple signals. AtRAC7/ROP9 may represent one of many adaptive ways the plant has evolved to integrate and reciprocally regulate the pathways triggered by different signals to best sustain its sessile lifestyle.

Recently, a pathway involving the receptor-like kinase FERONIA (FER), a ROPGEF, and a RAC/ROP protein was found to positively regulate auxin responses during the growth of roots and root hairs, while also acting as a negative regulator of ABA responses through the interaction of FER and ABI2 (Duan *et al.*, 2010; Yu *et al.*, 2012). This FER–ROPGEF–RAC/ROP module apparently also serves as an integrative point for auxin and ABA signalling. It will be interesting to determine whether AtRAC7/ROP9 is part of a signalling module that opposes that of FER–ROPGEF–RAC/

ROP or whether AtRAC7/ROP9 acts downstream of FER or other members of the kinase family (Hématy and Höfte, 2008; Cheung and Wu, 2011) but specifically downregulates auxin and upregulates ABA in other tissues or in other developmental processes not yet described.

Supplementary data

Supplementary data are available at JXB online.

Supplementary Fig. S1. (A) AtRAC7/ROP9p–GUS expression is activated in the root pericycle after auxin application. (B, C) The GFP–AtRAC7/ROP9 protein localizes exclusively to the plasma membrane (B), while the type I GFP–AtRAC5/ROP4 localizes to both the plasma membrane and the cytoplasm of transfected protoplasts (C).

Supplementary Fig. S2. (A, B) Primary root length (A) and lateral root density (B) are not affected in the AtRAC7/ROP9 RNAi lines. (C) Plants with reduced levels of AtRAC7/ROP9 mRNA display early flowering. (D–F) The seedling's responses to gibberellins, cytokinin, and brassinosteroids are not affected.

Supplementary Fig. S3. The activity of the AtRAC10/ROP11 promoter is repressed by ABA.

Supplementary Fig. S4. Location of auxin and ABA responsive elements in the AtRAC7/ROP9 promoter.

Supplementary Fig. S5. A proposed model for AtRAC7/ROP9 regulation of auxin and ABA pathways in *Arabidopsis*. Auxin signaling triggers downstream responses and simultaneously increases the expression of AtRAC7/ROP9 which in turn negatively regulates the auxin pathway. Increased AtRAC7/ROP9 expression will positively affect ABA signaling. ABA signaling represses AtRAC7/ROP9 expression in a negative feedback loop, repressing its own pathway and at the same time releasing the repression on auxin pathways.

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